Supporting Information

Stable small quantum dots for synaptic receptor tracking on live neurons

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Contents

Experimental Section

I. **Coating and characterization of small quantum dots (sQDs)**.

Chemicals and Instruments:

The 620 nm organic QDs (CZ600) were purchased from NN-Labs (Fayettiville, AR). The 655 nm organic QDs (Q21721MP) were purchased from Invitrogen. The 527 nm and 615 nm QDs were synthesized in the Smith lab at UIUC. The PEGylated alkanethiol ($HSC_{11}(EG)_4$-OH) and carboxyl PEGylated alkanethiol ($HSC_{11}(EG)_4$-COOH) were purchased either from Sigma-Aldrich (674508-250MG) or from ProChimia Surfaces (TH001-m11-0.2, TH003-m11-0.1). Streptavidin was purchased from Prospec-Tany TechnoGene Ltd., Ness Ziona, Israel). EDC (1-(3-dimehylaminopropyl)-3-ethylcarbodiimide·HCl) was purchased from ProteoChem (Cheyenne, WY, c1100-3x10mg). All other chemicals or solvents were purchased from Sigma-Aldrich unless indicated otherwise.

The absorption spectra were measured in a Perkin-Elmer Bio10 spectrometer. The sizes were measured by DLS using a Marvin Instrument Ltd. nano-ZS Zetasizer.

Coating sQD

The organic CdSe/ZnS core/shell QDs underwent coating exchange with the mixture of commercially available PEGylated alkanethiol and carboxyl PEGylated alkanethiol[1,2] (SI Fig. 1). In brief, the organic CdSe/ZnS QDs (25 μL, 2 mg/mL) were mixed with $HSC_{11}(EG)_4$-OH (90%, or 97.5%)/ $HSC_{11}(EG)_4$-COOH (10%, or 2.5%) in H_2O (degassed, 400 μL)/toluene (400 μL) with tetraethylammonium hydroxide (TEAH; 20% wt in H_2O) as base. The mixture was heated to 60°C for 4 hours under nitrogen (N_2). During the process, the transfer of QDs from organic phase into aqueous phase was observed by examining the phases for fluorescence under UV light. The mixture was washed with chloroform ($CHCl_3$) three times (400 μL, 200 μL, 200 μL). The aqueous solution was then passed through a self-packed DEAE anion exchange column (GE Healthcare, 17-0709-10). The neutral QDs were primarily washed off with water first. The negatively charged QDs were washed off with 30 mM PBS to get the COOH functionalized QDs (QD-COOH) in aqueous solution. The QD-COOH were further conjugated to streptavidin (SA) via EDC coupling method to give SA functionalized QDs (QD-SA).

For a typical QD-SA conjugation, 150 mL of QD-COOH solution (400 nM) in sodium borate buffer (20 mM, pH 7.4) was mixed with 15 mL of SA solution (10 mg/mL) in sodium borate buffer (20 mM, pH 7.4). To the mixture, 3 mL of EDC solution (freshly prepared in sodium borate buffer (20 mM, pH 7.4), 10 mg/mL) was added. The reaction mixture was stirred at room temperature for 2 hours. The conjugation solution was filtered through a centrifugal filter unit (100k cut-off, Amicon Ultra UFC510024), and washed for a minimum of 5 times with sodium borate buffer (10 mM, pH 8.3). The QDs were re-dissolved in PBS buffer (20 mM, pH 7.2), and filtered through a centrifugal filter unit (0.2 mm, Pall Life Sciences, ODM02C34). The QD-SA filtrate was collected and stored at 4°C.

Size measurement

Dynamic light scattering (DLS) was employed to measure the sizes of 620 nm emitting sQDs. Measurements were carried out with concentrated samples (>100 nM), after filtering through an Antontop 10 (0.02 μm, Whatman GmbH, 6809-1102) syringe filter before measurements. Each trace for autocorrelation was acquired for 15 s, and averaged over 12-15 runs per measurement. The autocorrelation function was analyzed using Zetasizer software (Ver. 7.02, Malvern Instruments Ltd.). Each DLS measurement resulted in an average QD diameter with a standard error of the mean. Finally, the size of sQDs was obtained by fitting the 10-30 DLS measurements to a Gaussian function.

Quantum yield and relative brightness of the sQD

The quantum yield of sQD was measured using Rhodamine 6G in ddH_2O (quantum yield 0.95)[3] as a standard using the equation $Q_x = \frac{A_s}{A_x} \times \frac{F_x}{F_s} \times \left(\frac{n_x}{n_s}\right)^2 \times Q_s$, where Q is the quantum yield, A is the absorbance at the excitation wavelength (488 nm); F is the area under the corrected emission curve, and n is the refractive index of the solvent. Subscripts s and x refer to the standard (Rhodamine 6G) and to the unknown (sQD-620), respectively. The spectra of absorbance and fluorescence of sQD-620 were measured using Agilent 8453 UV-Vis absorbance spectrophotometer (Agilent technologies) and PC1 spectrofluorimeter (ISS, Inc.), respectively. The quantum yield for the core of sQD-620 in organic solvent before surface coating was ~50%, which varies slightly between different batches according to NN-labs. After coating and coupling to SA, the quantum yield of the sQD-620 was 28%. For direct comparison of the brightness of commercial QDs (bQDs) and sQDs, single molecule fluorescent intensities of bQD-655 and sQD-655 were measured under the same illumination conditions. The average intensity of sQD-655 is 56% of that of the bQD-655. Therefore, with same core and same imaging conditions, the brightness of sQD was about half as bright as commercial QDs. Since we used 620 nm emitting sQD for most of our investigations, we also measured the single molecule fluorescent intensity of sQD-620 and it was about 1/3 (36.9%) as bright as Invitrogen QD-605.

Stability of the sQD:

The sQD solution (80-300 nM in PBS, pH 7.3) was stored at 4°C. In general, over 4 weeks, the sQD remained stable, showing no aggregation in solution. Sizes of small QDs from a fresh batch and from a 30 day old batch were measured by DLS. Both batches were 7.3 nm (SI Fig 2b-c). Single QDs were observed on coverslips (SI Fig.2d). When labeling neurons with sQDs, no significant increase in non-specific binding was observed (SI Fig. 2e).

During receptor tracking experiments, sample of neurons labeled with sQDs were kept and imaged at 37°C for 20 min ~ 30 min. We did not observe any obvious difference in behavior of the sQDs at the end of the experiment compared to at the beginning of the experiment. We have not tested the stability of sQDs at 37°C for longer time period. In theory, sQDs are more stable at room temperature than at 37°C. Therefore, sQDs can be used for experiments at 37°C and at room temperature, with a shelf life of one month when stored at 4°C.

II. Cell culture and labeling

HEK culture and labeling:

HEK 293 cells (from ATCC) were cultured in 35 mm glass bottom dishes in DMEM media supplemented with 10% FBS, 2 mM of L-Glutamine, 100 units/mL of penicillin, and 100 units/mL of streptomycin. Cells were transfected with GluA2-AP (0.4 μg/dish) and BirA-ER (1.6 μg/dish) using Lipofectamine 2000. After about 6 hours of transfection, cells were placed in fresh media supplemented with 10 μM of biotin, incubated overnight and imaged the next day. Before imaging, cells were incubated with 2 ml of blocking solution (1mg/mL BSA and 1mg/mL casein in optimum) for 1 h at 37°C, 5% CO_2. Cells were washed once with DPBS, and incubated in imaging buffer (1 mg/mL BSA and 1 mg/mL casein, and 0.5 nM of bQD-605 or 2 nM of sQD-615 in DPBS) for 10 min at room temperature. Cells were washed 3 times with DPBS before imaging. For testing sQD620-GBP labeling, the same protocol was used except that HEK cells were transfected with GluA1-GFP (0.4 μg/dish), incubated in normal media without biotin before imaging and labeled with 2 nM of sQD620-GBP.

Neuron culture:

Primary cortex and hippocampal cultures were prepared from E18-19 rats according to university guidelines as previous described[4] with the following modifications. Neurons were dissociated in 3 mg/mL protease and plated on 18 mm coverslips coated with 1 mg/mL poly-L-Lysine. Neurons were cultured at 37°C with 5% CO_2 in neurobasal media with B-27 supplement, 2 μM glutmax and 50 unit/mL penicillin and 50 unit/ml streptomycin. On 11-13 days in vitro (DIV), neurons were co-transfected with Homer1-mGeos-M (0.4 μg/coverslip) with GluA2-AP (0.4 μg/coverslip) and BirA-ER (0.4 μg/coverslip) by Lipofectamine 2000 transfection reagent. At 24 - 48 hours after transfection, the coverslips were transferred to warm imaging buffer (HBSS supplemented with 10 mM Hepes, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 2 mM D-glucose) for 5 min incubation and mounted onto an imaging dish (Warner RC-41LP). In the imaging dish, neurons were incubated in imaging buffer containing QDs and casein (~400 times dilution for bQDs, and ~80 times dilution for sQDs; stock solution purchased from Vector labs, SP-5020) for 5 min at 37°C and washed with 10 ml of imaging buffer with 1 μM biotin. Finally 1 mL of imaging buffer was added to the imaging dish that was subsequently mounted on the sample stage of the microscope.

Plasmids

Constructs GluA2-AP[5,6], BirA-ER[5,6] were described previously. Construct Homer1-dsRed was a gift from P. De Koninck (Université Laval Robert-Giffard, Québec, Canada).

To make Homer1-mGeos-M, the rat homer homolog 1 (Homer1; NM_031707.1) expression vectors were constructed using a N1 (Clontech-style) cloning vector housing an advanced EGFP variant mEmerald (wtGFP containing F64L, S65T, S72A, N149K, M153T, I167T, and A206K mutations). The following primers were used to amplify Homer1 and create an 18 amino acid linker separating Homer1 from the fluorescent protein:

NheI forward: GCG ACG CTA GCG CCA CCA TGG GGG AAC AAC CTA TCT TCA GCA CTC GAG CT

BamHI reverse: GCG CTG GAT CCC CTC CAC TCC CGG AGC CGC TAG AAC CGC TGG ACC CGC TGC ATT CTA GTA GCT TGG CCA AAT TAT CCC GCA A

The resulting PCR product and mEmerald-N1 were digested by the appropriate restriction enzymes, gel purified, and ligated to yield mEmerald-Homer1-N-18.

Upon sequence verification, mEmerald-Homer1-N-18, and a mEos2 variant, mGeos-N1 (mEos2 containing H62M mutation) were sequentially digested with BamHI and AgeI restriction enzymes, gel purified, and ligated to yield mGeos-Homer1-N-18.

The DNA used for transfection was prepared using the Plasmid Maxi kit (Qiagen, Valencia, CA).

III. Single molecule imaging on coverslips

Coverslip were coated with 95% PEG with 5% PEG-biotin according to the protocol[7]. Quantum dots (functionalized with SA) were diluted in PBS (100 pM for bQD, 1-2 nM for sQD) and flow into the channel on coverslip. For After 5-10 min, the coverslip was washed by PBS to remove the unbind QDs. Individual QDs immobilized on the coverslip were imaged later. For testing sQD-biotin, same procedure was used except for the coverslip was first incubated with 1 mg/ml SA for 5 min and washed with PBS to remove excess SA before applying sQD onto the coverslip.

IV. Optics and imaging

Experiments were performed with a Nikon Ti Eclipse microscope with a Nikon APO 100 X objective (N.A. 1.49). The microscope was equipped with the Perfect Focus System, which stabilizes the sample in z-axis. An Agilent laser system MLC400B with 4 fiber-coupled lasers (405 nm, 488 nm, 561 nm and 640 nm) was used for illumination. We use Elements software from Nikon for data acquisition. A back illuminated EMCCD (Andor DU897) was used for recording. For 3-D imaging, a cylindrical lens (CVI Melles Griot, SCX-25.4-5000.0-C-425-675) of 10 m focal length was inserted below the back of the objective. A motorized stage from ASI with a piezo top plate (ASI PZ-2000FT) was used for x-y-z position control. For fluorescence imaging, a quad-band dichroic (Chroma, ZT405-488-561-640RPC) was used and band-pass emission filter 525/50, 600/50, 710/40, 641/75 was used.

Tracking AMPAR:

The sample was first examined under bright-field and only coverslips with healthy neurons were used for experiments. After focusing on the sample, the Perfect Focus System was activated to minimize the sample drift in z direction. The samples were then scanned in the GFP channel (488 nm laser excitation, 525/50 emission filter) to locate transfected cells. A fluorescent image of the cells was taken for reference. To track the QD labeled receptors, 488 nm or 561 nm lasers was used for excitation in the Epi-fluorescence mode[8] with an appropriate band-pass filter for collecting the fluorescence. Detailed optical setup is listed in SI Table 1. Movies of 1000 frames were acquired with 50 ms exposure time.

	sQD 527	sQD 615	sQD 620	sQD 655	Invitrogen QD 705
Excitation light	488 nm	488 nm/ 561 nm	488 nm/ 561 nm	488 nm/ 561 nm	488 nm/ 561 nm
Emission filter	Chroma HQ 535/50m	Chroma ET 600/50m	Semrock BrightLine 641/75	Chroma D 655/40m	Semrock BrightLine 710/40

SI Table 1. Optical configurations for imaging QDs.

Super-resolution of post-synaptic density.

After the tracking experiment, the PALM[9] experiment was carried out on the same neurons. PALM was used for super-resolution of post-synaptic density. Post-synaptic protein Homer1 was used as the PSD marker and its C- terminus was fused to photoactivatable protein mGeos-M. A 100 ms 405 nm laser pulse was used to activate mGeos-M proteins from dark to green fluorescent state. The sample was then excited with a 488 nm laser and emission was collected with a 535/50 band-pass filter with 100 ms exposure time. The cycle was repeated for 200-300 times, collecting 4000-6000 frames. The z calibration was created following method described by Huang et al.[10] using fluorescent beads on a glass surface and applied to both tracking and PALM data.

V. Data analysis and visualization

For the single molecule coverslip test of QDs, individual quantum dots in a 7 by 7 pixel (pixel size is 160 nm) box with the intensity peak in the center was selected. The intensity count in the box was summed. To get the averaged intensity for QDs, the program detected all the QDs in a movie which were above a given threshold, and calculated their average intensity over the entire movie. The average was taken for all detected QDs.

QuickPalm[11], an ImageJ plug-in was used for analyzing single particle tracking data and PALM data. The x-, y-, z- coordinates of detected molecules/QDs were extracted from the image for further analysis. Image distortions from sample drift were corrected in QuickPalm by selecting reference points in the image.

For the tracking data, centroids of all the QDs were localized in all the frames and a map of all the places QDs visited were obtained. A Matlab code was used to recover the trajectories of the QDs. In brief, the code finds locations of QDs in time t, and searches for nearby QDs in time t+1 as the next point on the trajectory. In the 3-D single particle tracking experiment, the maximum displacement of a QD in one time step is set to be 1 μm; in 2-D single particle tracking of AMPARs on HEK cells, the max-displacement was set to 0.5 μm. Trajectory range was obtained by calculating the range of the trajectory in the x-, y-, and z-direction and using the maximum of the three parameters. The diffusion coefficients from the trajectories were calculated in Matlab by fitting the first 4 points of mean-square-displacement curve. As a result, the value of diffusion coefficient only reflects the displacements during short-time intervals. For the PALM data, positions of proteins detected in each frame are localized, cluster analysis was used for identifying synapses. To determine if a trajectory was synaptic, the centers of synapses were determined, and trajectories within 2 μm radius of each synapse were identified. For each of these trajectories, the average distance was calculated between the center of the nearest synapse and all points on the trajectory. Any trajectories with an average distance smaller than 0.75 μm were considered synaptic. Note that the localization precision, quantified by the localization error, of QD tracking depends on photon number of the QD and diffusion of the QD; and localization error for a moving QD is larger than that of a stationary QD. According to Michalet[12], the additional error caused by diffusion in the lateral direction (x and y), depends on diffusion coefficient D and the EMCCD exposure time t_E. In tracking experiment, the additional localization error caused by diffusion ($t_E = 0.05$ s, and D between 0.1 $\mu m^2/s$ – 1 $\mu m^2/s$) is 22%~50% of that of the same QD at stationary state.

The visualizations of post-synaptic densities and sQD labeled AMPAR tracking trajectories were created using VMD 1.9.2[13], a widely-used program for visualizing and analyzing macromolecular structures and molecular dynamics (MD) simulation trajectories. VMD is optimized for dealing with large data sets containing millions of particles and thousands of trajectory frames, as encountered in the current study.

The PALM data were loaded into VMD using a short script that assigned 3-D coordinates to a set of particles with uniform per-particle radius and density weighting factors. Post-synaptic densities were approximated using the QuickSurf representation[14] in VMD 1.9.2, which has recently been extended for flexible display of non-atomic particle systems such as cellular models[15], very large particle counts, and high-quality ray tracing of large complexes[16]. The single particle tracking data were also loaded into VMD as a set of particles with 3-D coordinates, and uniformly assigned radii and density weights, but they were also sorted temporally into a sequence of trajectory frames similar to an MD trajectory. Unlike typical MD trajectories which usually contain a constant number of particles in all frames, the particle counts in each frame of the tracking data varies as a result of blinking or photobleaching. The varying number of particles in each trajectory frame was handled by creating a fixed number of particles in all frames sufficiently large to store the largest trajectory frame, and incorporating atom selections to select only the particles actually used. The tracking data were visualized as a sequence of frames as shown in the SI movies, or all at once in a superposition of multiple time steps, as shown in Fig 3 (a) and (d).

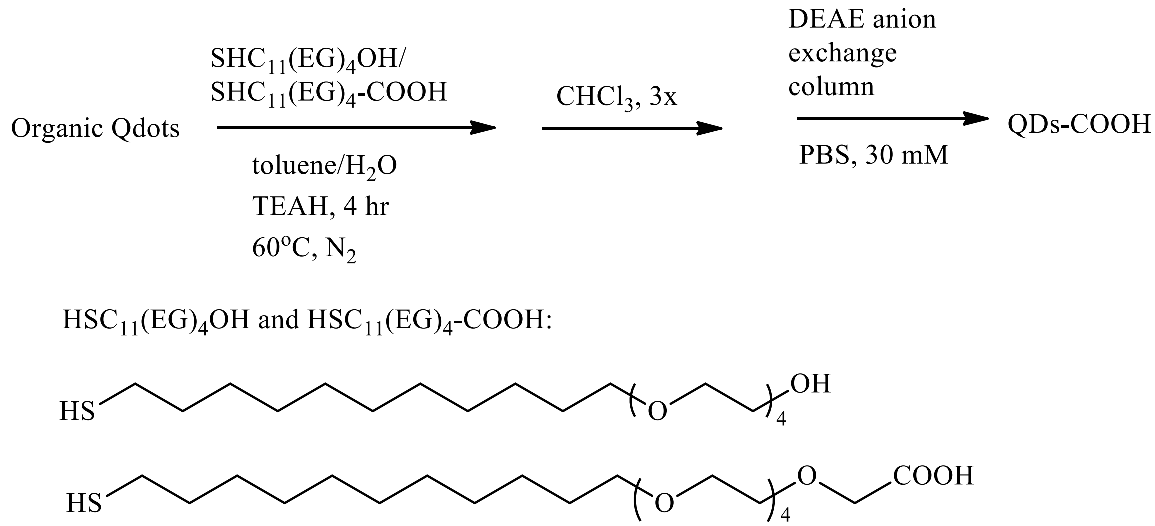

Figure S1: Coating procedure for small quantum dots.

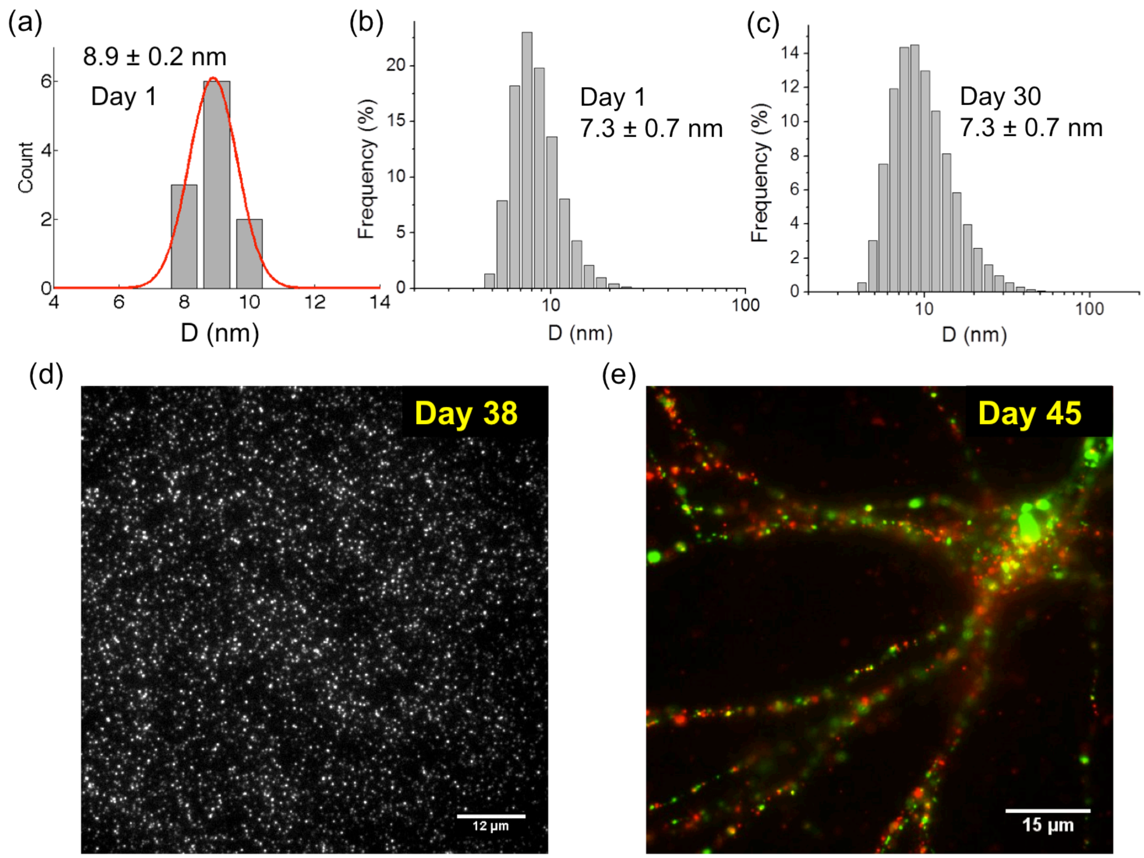

Figure S2: Characterization of 620 nm emitting small quantum dots (sQD) functionalized with streptavidin (SA).
(a) The size of sQD-620-SA (with 10% COOH) was 8.9 ± 0.2 nm. The results were obtained by calculating the means of 11 DLS measurements taken on the day of synthesis. The error is the standard error of the mean. Note: DLS measurement is described in the SI method.
(b) Single DLS measurement of sQD-620-SA (with 2.5% COOH) was performed on the day of synthesis, the size from one DLS measurement was 7.3 ± 0.7 nm. The error is the standard deviation. Note that sQD620-SA with 2.5% COOH has fewer SA per QD, resulting in a size smaller than sQD620-SA with 10% COOH.
(c) Single DLS measurement of sQD-620-SA (with 2.5% COOH) was performed 30 days after synthesis. The sQD-620-SA used were stored at 4°C. The size from one DLS measurement was 7.3 ± 0.7 nm. The error is the standard deviation.
(d) Fluorescent image of sQD-620-SA binding to biotins on a PEGylated coverslip. The sQD-620-SA used had been stored at 4°C for 38 days.
(e) Fluorescent image of biotinylated AMPA receptors on live neurons specifically labeled with sQD-620-SA. The sQD-620-SA used had been stored at 4°C for 45 days. In the image, Homer1-mGeos are shown in green, and sQDs in red. Most of the sQDs are co-localized on the transfected neuron; only a few sQDs are non-specifically bound elsewhere.

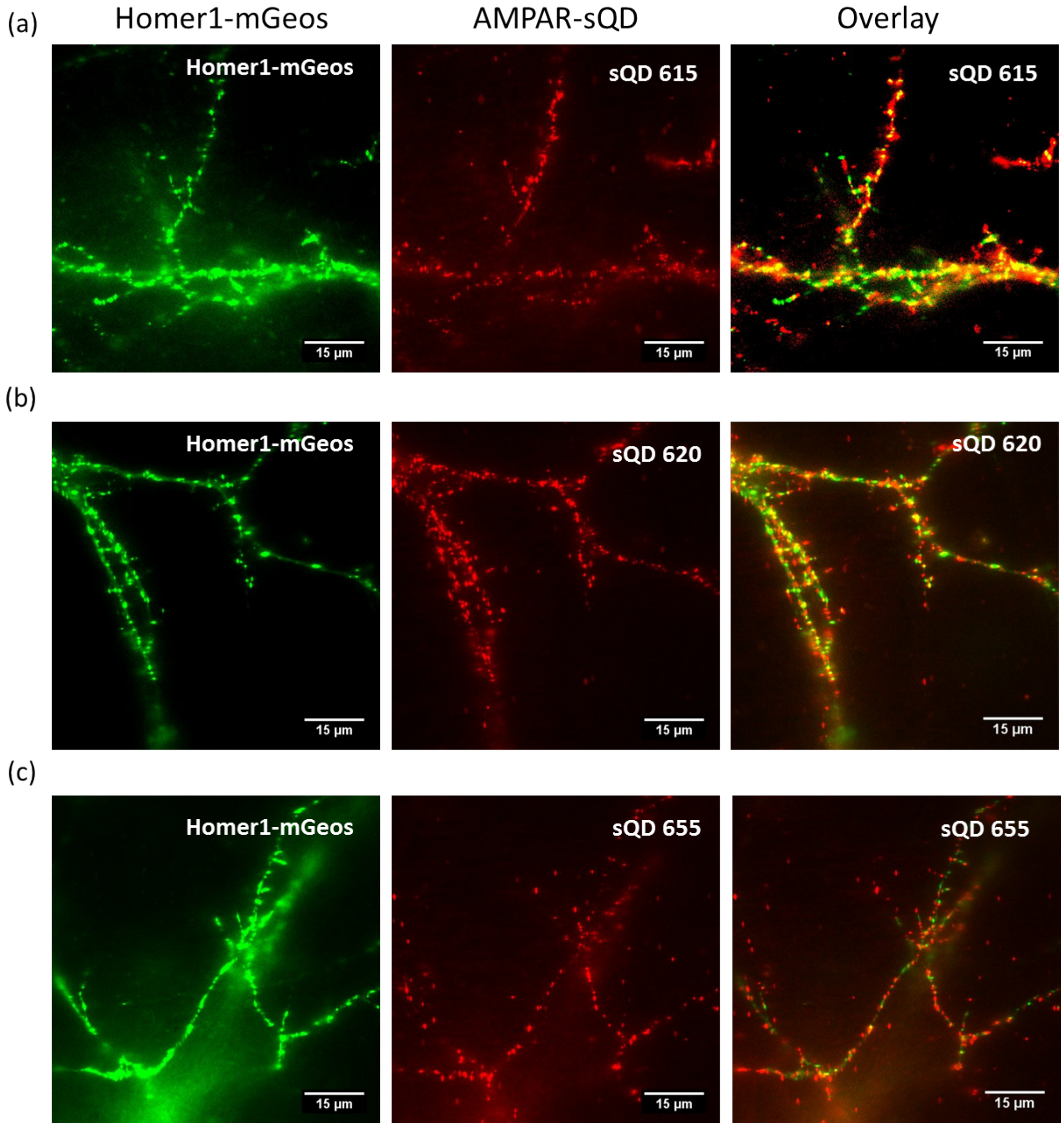

Figure S3: Specific labeling of biotinylated AMPA receptors (AMPARs) on the surface of live neurons with small quantum dots (sQDs), functionalized with streptavidin (SA).

Cultured neurons (from hippocampus or cortex) were co-transfected with Homer1-mGeos, GluA2-AP and BirA-ER. Homer1-mGeos is shown in green; small QDs of different emission wavelengths (615 nm (Laboratory-made), 620 nm (NN-Labs), 655 nm (Invitrogen)) were used to label the biotinylated AMPARs (shown in red); an overlay is shown at right, showing the overlap. The entire field of view is full of neurons, but only the neurons shown in green are transfected and co-expressing the post-synaptic density protein Homer1 (green) and biotinylated AMPAR. Therefore, sQDs (red) found on the particular neuron demonstrate specific binding of the sQDs to biotinylated AMPARs. The amount of co-localization varies from neuron to neuron (compare the upper region with the bottom region of the neuron in the overlay image of sQD-

615) and between different sQDs sources (compare the sQD620 NN-Labs vs. sQD615 laboratory-made) and may depend on the exact focal plane used in the microscopy.

(a) Fluorescent images of a hippocampal neuron expressing Homer1-mGeos (left) and labeled with sQD-615-SA (middle). An overlay image (right) shows the labeling specificity. Note: The exact intensity for the red and green channels was independently optimized for the upper and lower regions of the neurons, to balance the uneven brightness of the original images.

(b) Fluorescent images of a hippocampal neuron expressing Homer1-mGeos (left) and labeled with sQD-620-SA (middle). An overlay image (right) shows the labeling specificity

(c) Fluorescent images of a cortical neuron expressing Homer1-mGeos (left) and labeled with sQD-655-SA (middle). An overlay image (right) shows the labeling specificity.

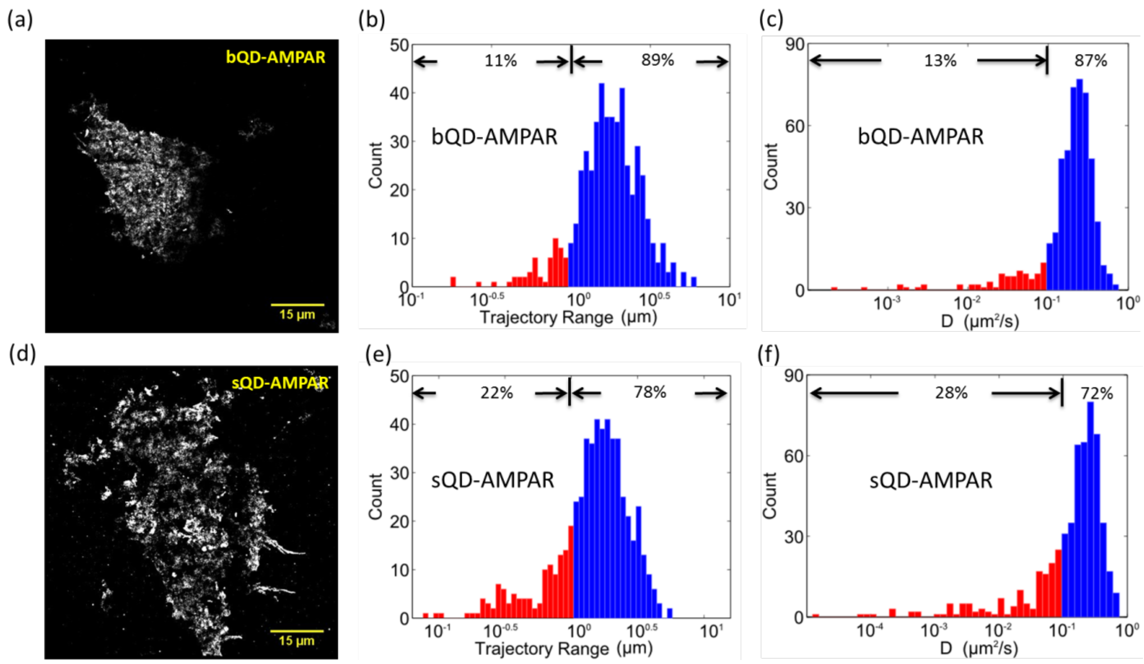

Figure S4: Diffusion of AMPA receptors (AMPAR) expressed in HEK 293 cells.
HEK 293 cells were transfected with GluA2-AP and BirA-ER and labeled with either bQDs (a-c) or sQDs (d-f) functionalized with streptavidin (SA).
Positions of AMPARs labeled with QDs were obtained by single particle tracking over 50 s time period in 2-D. Positions of all AMPARs diffusing on the cell membrane during a 50 s time period are shown in (a) for Invitrogen QD 605-SA (i.e. bQD) labeled AMPARs and (d) for sQD-615-SA labeled AMPARs. (b) and (e) are histograms of trajectory range for AMPARs labeled with bQD and sQD respectively. They show that most of the QD-AMPARs were free to diffuse in a large range. (c) and (f) are histograms of diffusion coefficients for AMPARs labeled with bQD and sQD respectively. Although AMPARs labeled with bQD and sQD are slightly different in their diffusion behavior, in both cases, the majority of AMPARs diffused in large range with coefficient larger than 0.1 $\mu m^2/s$.

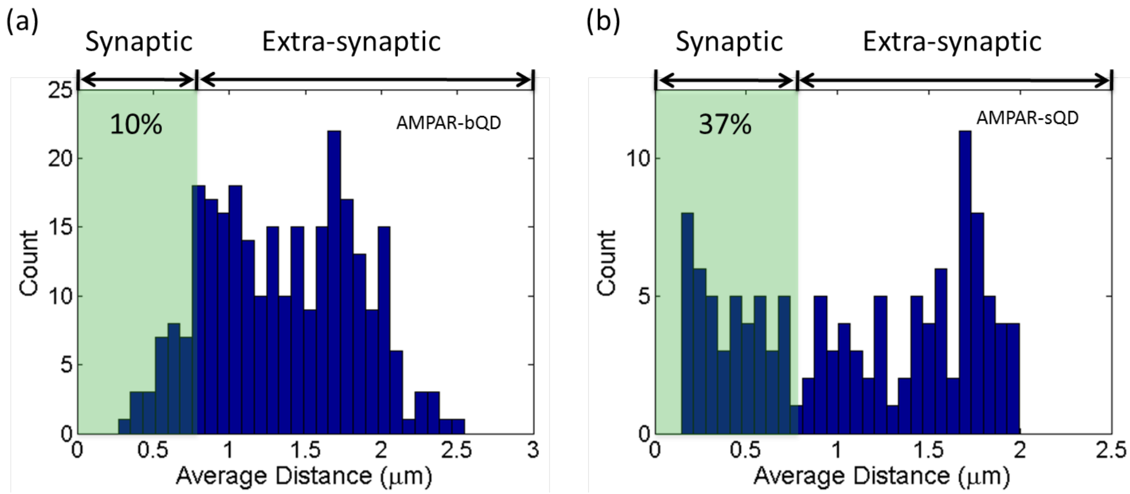

Figure S5. Average distance of AMPA receptors (AMPARs) diffusion trajectory to the center of nearby synapses.

(a) A histogram of average distances of AMPAR trajectories to the center of nearby synapse. The average distance between a trajectory and the center of a nearby synapse were calculated. Statistics were done for all the trajectories within 2 μm to the center of any synapses. For Invitrogen QD-655-SA, only 10% of the trajectories were synaptic (within 0.75 μm to the center of synapses).

(b) Same scheme as in (a) but for AMPARs labeled with sQD-620-SA. A total of 37% of the trajectories were synaptic (within 0.75 μm to the center of synapses). Note that many extra-synaptic sQD-AMPAR diffuse in small ranges (trajectory range < 1 μm). Whether these regions are synapses not labeled with mGeos-Homer1, or some other regions, is unclear.

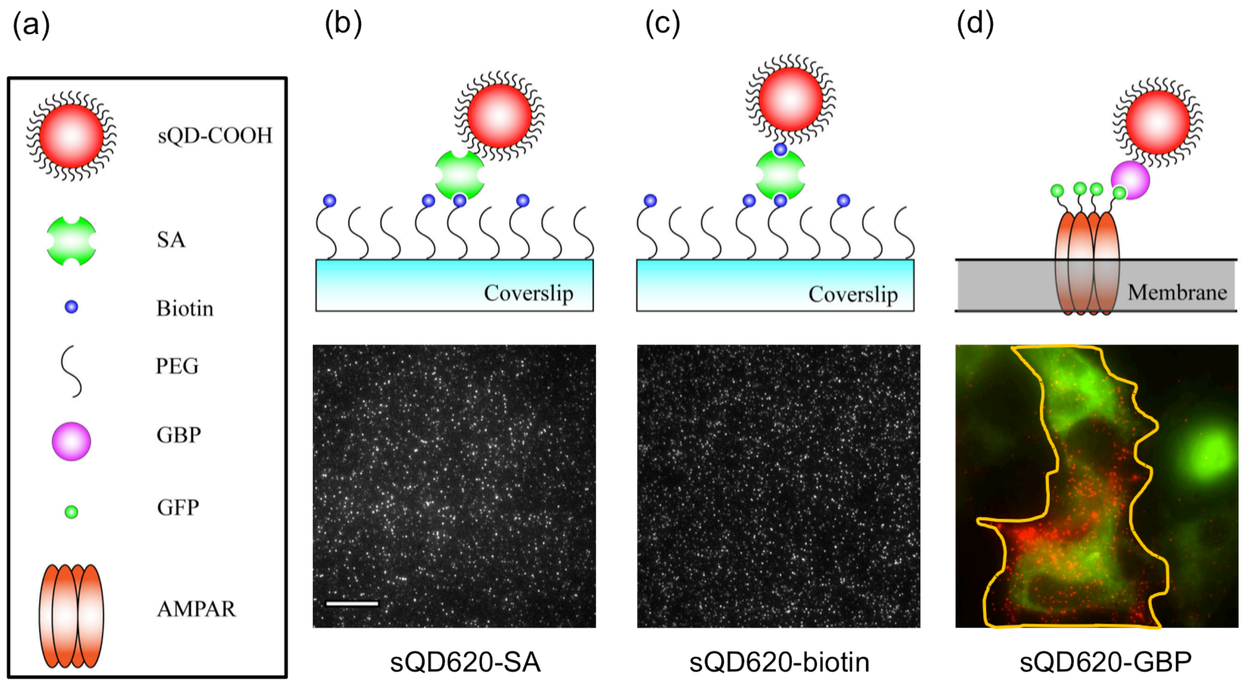

Figure S6: Small QDs functionalized with different biomolecules.
(a) Schematic drawings for sQD-COOH, streptavidin (SA), biotin, PEG, GFP binding protein (GBP)[17], GFP and AMPA receptor (AMPAR).
(b) (Top) Schematic drawings of sQD620-SA bound to a coverslip coated with 5% biotin-PEG and 95% PEG. The sQD620-SA then binds to the biotin molecules. (Bottom) The fluorescent image of sQD620-SA bound to a PEGylated coverslip.
(c) (Top) Schematic drawing of sQD620-biotin bound to a coverslip coated with 5% biotin-PEG and 95% PEG. SA was immobilized on the coverslip by reacting with the PEG-biotin and then the sQD620-biotin was added. (Bottom) The fluorescent image of sQD620-biotin bound to a PEGylated coverslip.
(d) (Top) Schematic drawing of the labeling of sQD620-GBP to GluA1-GFP on the surface of an HEK 293 cell. GBP is a fragment from a llama single chain antibody that binds to GFP specifically. (Bottom) The green signal represents GluA1-GFP, and red signal represent sQD620-GBP. The cell contour is shown in yellow. sQD620-GBP only bound to cells expressing GluA1-GFP demonstrate the labeling were specific.

Supporting Movies

Movie 1. Movie of AMPARs labeled with commercial QDs (bQDs) diffusing between synapses. The blue clusters depict localizations of post-synaptic marker Homer1-mGeos-M, and the red dots are localizations of bQD-labeled receptors at different time points on trajectories. One region is enlarged to show the AMPAR diffusing along the dendrite between two synapses.

Movie 2. Movie of AMPARs labeled with small QDs (sQDs) diffusing in the synapse. The blue clusters depict localizations of post-synaptic marker Homer1-mGeos-M, and the red dots are localizations of sQD-labeled receptors at different time points on trajectories. One region is enlarged to show the AMPAR diffusing in the synapse.

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